prosecution, Applicants have amended claim 2 to place the claim in more traditional Markush format.

Claim 4 has been rejected for recitation of "a part or parts or the...." Claim 4 has been amended to correct this apparent typographical error and instead recite "a part or parts of the...."

Claim 10 has been rejected for recitation of "The method claim 1...." Claim 10 has been amended, as indicated above, to properly recite, "The method of claim 1...." Claim 10 has been further amended pursuant to the suggestions of the Examiner to clarify the mete and bounds of the claim. Claim 11 has been similarly amended.

Claim 13 is believed to appropriately define the invention, in view of the above-indicated amendments.

As the above amendments to the claims address the rejections of the Examiner and fully define and clarify the claims, withdrawal of the rejections under 35 U.S.C.§112, second paragraph are respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claim 2 has been rejected under 35 U.S.C. §112, first paragraph as lacking support for recitation of "nonsense" mutations. "Nonsense" mutations are fully supported by the specification on page 3, lines 13-14, wherein a "nonsense" mutation is defined as one causing truncation of the protein, i.e. the inappropriate creation of a stop codon.

"Nonsense" mutations are further supported in the specification by the data of Table 2, wherein mutations creating nonsense mutations in p53 have been shown. See amino acids residues 204, 317, 331 and 342, wherein the respective amino acid residues were replaced with stop codons, thus creating nonsense mutations.

Claims 1-11 have been rejected under 35 U.S.C.§112, first paragraph for lack of enablement. More specifically, the Examiner maintains that claims encompass the independent use of mutation and node status in prognosis, for which the specification is not enabled.

Claim 1 has been amended to more clearly define that the results obtained in steps c)(i) and c(ii) are used in combination, i.e. both node status and mutational analysis, to make the prognosis. Applicants believe this amendment fully addresses the issue of enablement raised by the Examiner.

Rejections under 35 U.S.C. §103

Claims 1-8 remain rejected under 35 U.S.C.§103, as being obvious over Elledge et al. in view of Callahan and Hartmann et al. In the rejection the Examiner raises the following points.

1) Firstly, the Examiner notes on page 6, of the Final Office Action, that the claims as currently written recite "analyzing the

DNA sequence..." rather than "sequencing the DNA." Thus, the use of SSCP is encompassed by the claims.

2) The Examiner asserts that Elledge et al., in fact, sequence samples to determine p53 mutations.

3) On page 7, of the Final Office Action, the Examiner indicates that there is no evidence that DNA sequencing is superior to SSCP due to inefficiency of the SSCP analysis.

4) The Examiner further states that any unexpected advantage must be disclosed at the time of filing.

5) The Examiner asserts Elledge et al. found the combination of p53 mutation and node negative status was a poor prognostic indicator with a higher risk of relapse. Based on this, the Examiner asserts that Elledge et al. teach that p53 mutation combined with node negative status indicate a prognosis of relapse and provide guidance that adjuvant therapy is required.

6) The Examiner maintains that it would be obvious to combine nodal status analysis of Callahan with the mutational analysis of Elledge et al.

Applicants respectfully traverse this rejection and will address each point raised by the Examiner, in turn. Firstly, the Examiner asserts that the claims recite "analyzing the DNA sequence..." thus encompassing the use of SSCP. Claim 1 has been amended, as indicated above, to more clearly recite in steps a) and b) - a) "determining the

nucleotide sequence of the complete coding region of the p53 protein; b) analyzing the nucleotide sequence determined in step a) for the presence of mutations." Support for determining the "nucleotide sequence of the complete coding region of p53" is found in the experimental section of the specification, wherein the complete nucleotide sequence was sequenced. Thus, claim 1 has been amended to more clearly recite that the present invention includes a step of sequencing the samples.

As indicated in point 2) above, the Examiner asserts on 6 of the Final Office Action that, "Elledge et al. does sequence samples to determine p53 mutations...." Page 97, left, 1st paragraph through the right column, 1st paragraph, of Elledge et al. states,

> We focused our search for p53 mutations on exons 5 through 9 because the majority of p53v mutations in tumors have been found in this region (30). This region of the gene is highly conserved in evolution (31), which reflects its functional significance. Three segments of the p53 sequence in this region were amplified by DNA PCR. These segments encompassed exons 5 and 6, exon 7, and exons 8 and 9. The amplified fragments were examined by SSCP analysis...[left column, final paragraph] An example of SSCP analysis for exons 8/9 is seen in Figure 1. Tumor DNA samples were run under both non-denaturing and denaturing conditions. Under denaturing conditions, the DNA is separated into single strands. An abnormally migrating band, representing a mutation, is seen in the far right lane. This abnormal band was cut from the gel, and the DNA cloned and sequenced.

Thus, it is clear from the disclosure of Elledge et al. that the entire p53 region is not sequenced and not all samples were sequenced in any

way. The only sequencing that was done was a limited partial sequence analysis of those mutations, which were identified using SSCP.

On the bottom of page 6, spanning page 7 of the Office Action, the Examiner asserts that there is no evidence that DNA sequencing is superior to SSCP due to inefficiency of the SSCP analysis. (Point 3, above). The degree of mutations detected using SSCP will depend on the conditions used, and on the particular gene being analyzed. For p53, specifically, it is now known that SSCP is inefficient. As evidence of the inefficiency of SSCP in detecting mutations of p53, enclosed herewith is an article by Tolbert et al. wherein it is clearly stated that, "p53 immunoreactivity and single strand conformational polymorphisms analysis often fails to predict p53 mutational status." Mod. Pathol. 12(1) 54-60 (1999). As further stated on page 60 of Tolbert et al. the sensitivity of SSCP for detecting mutations of p53 is as low as 62%. Thus, Applicants have clearly evidenced the inefficiency of SSCP in determining sequence mutations of p53.

In point 4) listed above, the Examiner asserts that any unexpected advantage must be disclosed at the time of filing. Applicants respectfully request that the Examiner explain the basis for this assertion. M.P.E.P. §716.02(f), citing to In re Chu, 66 F.3d 292, 298-299, 36 U.S.P.Q.2d 1089, 1094-95 (Fed. Cir. 1995) clearly instructs,

> We have found no cases supporting the position that evidence or arguments traversing a §103 rejection must be contained within the specification. There is no logical support for such a proposition as well, given that obviousness is

> determined by the totality of the record including, in some instances most significantly, the evidence...proffered during ex parte prosecution.

It was further held in Ex parte Sasajima, 212 U.S.P.Q. 103,104-5 (Bd. Pat. App. and Interfer. 1981), that evidence relating to initially undisclosed relative toxicity of claimed pharmaceutical compounds must be considered. Nowhere is it indicated that an unexpected advantage over a prior art reference must be disclosed at the time of filing. Both the rules and the M.P.E.P are clear in that an Applicant may rely on an unexpected advantage not disclosed in the specification to establish the non-obviousness of an invention.

Previously known methods, such as those of Elledge et al., for using p53 mutational status as a prognostic tool, had the following two prejudices.

a) It was considered that one could disregard mutations outside the conserved region when using p53 as a prognostic marker.

b) It was believed that the determination of p53 mutational analysis could greatly simplified by using methods such as SSCP.

The present inventors have found distinct weaknesses associated with the presumptions (a) and b) above), of the prior art methods and developed an improved prognostic method, which relies on full mutational sequence analysis of p53. The advantages associated with the present method exist independent of whether nodal status is also

used, although combining the full p53 sequence analysis with nodal status results in an even more improved prognostic tool. The advantages associated with the full sequencing of the p53 gene are evidenced by the enclosed article by Kressner et al., J. Clin. Oncol. 17(2) 593-599 (1999)(co-authored by the present inventor), wherein it is shown that the relative hazard for a mutation is 1.7, independent of where the mutation occurred. Further shown in Table 3 of the article, the relative hazard for patients with a missense mutation is 1.45 and for other than a missense mutation 2.36. Kressner et al. further teach in Figure 1, that most missense mutations are localized in the central conserved areas. However, most of the mutations which are other than missense are localized to end portions of the gene. See page 597, right column, of Kressner et al. These mutations would have been missed using the SSCP analysis of Elledge et al. wherein only the conserved region was analyzed. See page 97, left column, of Elledge et al.

Although, the Figures in Kressner et al. regarding the relative hazards, discussed above, do not incorporate nodal status they demonstrate unexpected advantages associated with mutational analysis alone of the complete p53 coding region. Points 5) and 6) above, regarding nodal status, in no way suggest the advantages associated with full p53 sequence analysis. As such, points 5 and 6) do not render the present invention obvious.

In summary, the methods of the prior art, which used p53 mutational analysis as a prognostic tool, were based on two premises, a) mutations outside the conserved region of p53 could be disregarded; and b) p53 mutational analysis could greatly simplified by using methods such as SSCP.

The present invention has unexpected advantages over the prior art because, as evidenced by Tolbert et al., SSCP analysis is inefficient in identifying mutations in p53. Elledge et al. discloses only limited sequencing of portions of the p53 gene from samples selected using SSCP. As such, the method of Elledge et al. will miss a significant portion of the mutations which may be present. The invention of the present claims, as amended, require the full sequencing of the entire p53 coding region from each patient sample. One skilled in the art would not have thought to use full sequencing of the p53, given that SSCP analysis, which is much simpler, was believed to be sufficient. This is evidenced by page 60, left column, second paragraph of the Tolbert et al. article, which indicates that prior to the present invention, it was thought that SSCP analysis had a sufficiently high sensitivity for mutational analysis. However, only by sequencing the entire coding region of the p53 gene of every patient sample, will all mutations be detected. Thus, the present invention is distinguished from Elledge et al. and provides unobvious advantages over Elledge et al. Finally, contrary to the assertion of the Examiner, an advantage

of an invention over a prior art reference need not be disclosed at the time of filing.

With regard to the secondary reference, there is no suggestion in Callahan of sequencing the entire p53 gene and optionally combining such sequence information with nodal status, nor is there any suggestion in Callahan of the advantages associated with such a method. As such, Callahan does not overcome the deficiencies of Elledge et al. The present invention is therefore not obvious over Elledge et al. in view of Callahan.

Claims 9-11 and 13 were further rejected as being obvious over the additional references of Hedrum et al. Applicants believe the arguments discussed above, regarding claims 1-8 are equally applicable to the rejections of claims 9-11 and 13. In addition, the additional reference of Hedrum et al. in no way overcomes in the deficiencies of Elledge et al., Callahan and Hartmann et al. as discussed above. As such, the invention of claims 9-11 and 13 are not obvious over the cited prior art.

As the above-presented amendments and remarks address and overcome the rejections of the Examiner, withdrawal of the rejections and reconsideration and allowance of the claims are respectfully requested. Should the Examiner have any questions regarding the present application, she is requested to contact MaryAnne Liotta, PhD (Reg. No. 40,069) in the Washington DC area, at (703) 205-8000.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By MaryAnne Kolb (Reg No 40,067)
Gerald M. Murphy, Jr.
Reg. No. 28,977
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000

attachment: Talbert et al., Mod. Pathol. 12(1) 54-60 (1999)
Kressner et al., J. Clin. Oncol. 17(2) 593-599 (1999)

GMM/MAL

Prognostic Value f *p53* Genetic Changes in C lorectal Cancer

By Ulf Kressner, Mats Inganäs, Sara Byding, Ingrid Blikstad, Lars Påhlman, Bengt Glimelius, and Gudrun Lindmark

Purpose: To explore whether there is a linkage between different mutations in the *p53* gene in primary colorectal cancer and the risk of death from colorectal cancer in a large group of patients with long follow-up. We also compared a complementary DNA-based sequencing method and an immunohistochemical (IHC) method for detecting p53 protein overexpression in colorectal cancer.

Materials and Methods: The entire coding region of the *p53* gene was sequenced in 191 frozen tumor samples collected from January 1988 to November 1992. RNA was extracted and synthesized to cDNA. *p53* was amplified by the polymerase chain reaction, and the DO-7 monoclonal antibody was used in the IHC assessments.

Results: Mutations were detected in 99 samples (52%) from 189 patients. There was a significant relationship between the *p53* mutational status and the cancer-specific survival time, with shorter survival time for patients who had *p53* mutations than for those who did not ($P = .01$, log-rank test). Mutations outside the evolutionarily conserved regions were associated with the worst prognosis. Multivariate analysis showed that the presence of *p53* mutations was an independent prognostic factor (relative hazard, 1.7, $P = .03$). There was no significant relationship between overexpression of *p53* protein, as determined by IHC analysis, and cancer-specific survival.

Conclusion: Mutational analyses of the *p53* gene, using cDNA sequencing in colorectal cancer, provide useful prognostic information. In addition, cDNA sequencing gives better prognostic information than IHC assessment of *p53* protein overexpression.

J Clin Oncol 17:593-599.

THE DUKES CLASSIFICATION SYSTEM and similar systems based on the extent of tumor spread (eg, Astler-Coller and the tumor-node-metastasis classification) represent the most commonly used staging procedures in colorectal cancer.[1,2] The tumor stage provides prognostic information but not to the extent that it can be used to meet current requirements for differentiated therapy. The toxicity and expense of additional therapy can only be justified in the subset of patients at high risk for relapse. A large number of potentially clinically useful prognostic factors have been identified,[3] among them, mutational status of the *p53* gene.

The *p53* suppressor gene, located on the short arm of chromosome 17,[4] encodes a 53-kd nuclear phosphoprotein that regulates the cell cycle.[5,6] Mutations in this gene constitute some of the most frequently occuring genetic changes found in human malignancies.[4,5] They are thought to be a late development in the adenoma-carcinoma sequence in colorectal cancer.[7] It has been reported that *p53* mutations seem to be associated with poor prognosis in colorectal cancer.[8-11] However, conflicting results have also been claimed.[12] Overexpression of the *p53* protein is detectable in 30% to 70% of the tumors, using immunohistochemical (IHC) methods. In a great majority of studies, *p53* protein overexpression has been used as a surrogate marker for *p53* mutations, an assumption that is not entirely correct,[13] although it may sometimes be justified for practical and economic reasons. In some studies,[14-17] *p53* protein overexpression has been shown to correlate with patient survival, a finding that has not been observed in other studies.[10,18-22]

The purpose of this study was to explore and to determine whether there is a valid association between the mutations in the *p53* gene, as determined by cDNA sequencing, and the risk of death from colorectal cancer, as studied in a large group of patients with long follow-up. In addition, we investigated the relationship between observed mutations in the *p53* gene and the overexpression of the *p53* protein, as detected by IHC analysis. We also evaluated whether there is any association between cDNA sequencing and serum *p53* antibody levels in preoperative serum samples.

MATERIALS AND METHODS

Patients

Tumor samples were collected from 194 nonselected patients resected for colorectal cancer in the Uppsala and Falun counties in Sweden between January 1988 and November 1992. One hundred ninety-one meticulously dissected, fresh-frozen samples were available for analyses. Two patients contributed samples from two different

From the Departments of Surgery and Oncology, University Hospital, Uppsala; Pharmacia Biotech AB, Uppsala; and Department of Surgery, Umeå University Hospital, Umeå, Sweden.

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Address reprint requests to Ulf Kressner, MD, PhD, Department of Surgery, Uddevalla sjukhus, 451 80 Uddevalla, Sweden; email ulf.kressner@nu.alvsborg.se.

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tumors. Subsequently, one tumor sample from each patient was included in the survival analyses. Serum samples were collected before surgery from 90 patients (47%). The age and sex distribution, Dukes stage, and tumor differentiation are listed in Table 1. One hundred sixty-seven patients (87%) were resected for cure. In the remaining 24 patients, distant metastases were detected during surgery; consequently, these patients underwent a palliative resection. This group was classified as Dukes stage D. Twenty-eight of the patients (43%) with rectal cancer received preoperative radiotherapy. No patient received any postoperative adjuvant radiotherapy or chemotherapy. At follow-up in January 1997, 74 patients (39%) had died from cancer or from other causes with known tumor burden. Twenty-eight patients (15%) died from other causes without any indication of tumor relapse. No patient was lost to follow-up. The median survival time of the 89 patients alive at follow-up was 87 months (range, 51 to 106 months). Routine biopsy samples were taken from each tumor for histopathologic classification. The tumors were graded according to the World Health Organization classification[23] and staged according to the Dukes classification system.[1]

Sequence-Based Analyses of p53

cDNA sequencing was performed essentially as described by Sjögren et al,[13] with some minor modifications of polymerase chain reaction (PCR) primers, fluorescent label, and analysis platform.

RNA was prepared from the frozen tumor samples, under stringent conditions, to avoid degradation and contamination. This procedure was followed by an enzymatic synthesis of cDNA, using RNA as template. *p53* was amplified from the tumor cDNA by PCR, using four overlapping primer pairs covering the entire coding region of the *p53* gene. With one of the primers (in each primer pair) modified with a biotin molecule, biotin-labeled PCR products were generated, thus facilitating solid sequencing.[24] Manifold (solid-phase) sequencing was performed essentially as described by Lagerkvist et al.[25] The sequencing products generated were analyzed using an automated laser fluorescence sequencer (ALFexpress; Pharmacia Biotech, Uppsala, Sweden). The sequence was finally compared with the wild-type *p53* sequence, using prototype software program p53 SB Decipher, version 1.00 (Pharmacia Biotech). Nucleotide changes that had an impact on the protein were considered to be mutations. Each identified mutation was verified by sequencing an entirely new PCR product from the corresponding cDNA. Further confirmation was obtained by analysis of the neighboring PCR fragment when the mutation was located in an overlapping segment.

IHC Analysis of Overexpression of p53 *Protein*

p53 protein overexpression (mutated and wild-type) was evaluated by use of an IHC method in 190 tumor samples.[23] In a previous study, we investigated four different antibodies for overexpression of the *p53* protein and compared the homogeneity in multiple biopsy samples collected meticulously and randomly from each tumor.[26] A tumor section with positive staining by the the anti-*p53* antibody was clearly homogenously stained, ie, virtually all tumor nuclei were positively stained. We concluded that DO-7 did not show intratumor heterogeneity; therefore, we selected DO-7 for further analyses. We lack information about *p53* IHC analysis from one of the tumors. Briefly, DO-7 monoclonal antibody was used, in combination with biotinylated horse-anti-mouse IgG antibody as secondary antibody, followed by incubation with Vectastain ABC ELITE reagent (Vector Laboratories, Burlingame, CA) and development with 3-amino-9-ethylcarbazole.

Analysis of Serum p53 *Antibody Levels*

Serum samples were collected before surgery from 90 patients. The serum samples were stored at −70°C until analyzed. Anti-*p53* was measured using an enzyme-linked immunoadsorbent assay (Dianova, Hamburg, Germany), described in detail elsewhere.[27]

Statistical Analyses

The Cox proportional hazards model was used in both the univariate and the multivariate survival analyses.[28,29] Cancer-specific survival curves were constructed using the Kaplan-Meier method, and differences between curves were tested using the log-rank test. Censored cases in the analyses were patients who died from other causes with no known residual cancer disease. The χ^2 test was used to test for differences in distribution between groups. The statistical software Statistica, version 5.0 (StatSoft Inc, Tulsa, OK), was used for the analyses. *P* values of less than .05 were considered statistically significant.

RESULTS

Changes in p53

The *p53* mutational status for 189 colorectal cancers out of 191 was determined by cDNA sequencing. From the remaining two samples, no PCR products were generated. Mutations were detected in 99 samples (52%) from 189 patients. One hundred seven genetic changes were found (84 missense mutations, 16 deletions, three insertions, and four stops). Eight samples displayed two different mutations (five tumors had two missense mutations, one tumor had one deletion and one insertion, one tumor had two deletions, and one tumor had an insertion and one missense mutation). *p53* mutations were found throughout the entire protein coding region of the gene. Seventy-nine (74%) of the 107 mutations were located in the conserved regions of *p53* (Fig 1). Most of the missense mutations (83%) occurred inside conserved regions, whereas other genetic changes more frequently occurred outside. There was no difference in the frequency of mutations within the Dukes stages (Table 1). Tumors in the distal colon and rectum contained more frequent mutations than tumors in the proximal colon.

Comparison of p53 *cDNA Sequencing and* p53 *IHC Analysis*

Overexpression of *p53* protein was demonstrated in tumors from 92 (48%) of the 190 patients.[23] In the 188 cases in which a comparison was possible, there was a concordance in the results of the cDNA sequencing and the IHC analysis in 140 tumors (74%; Table 2).

Twenty-eight (29%) of the tumors that were positive by cDNA sequencing were negative on IHC analysis. This discrepancy was observed in three (75%) of four tumors with mutations that created premature stop codons and in nine (64%) of 14 tumors with deletions, but was much more uncommon in tumors with missense mutations (15 of 84,

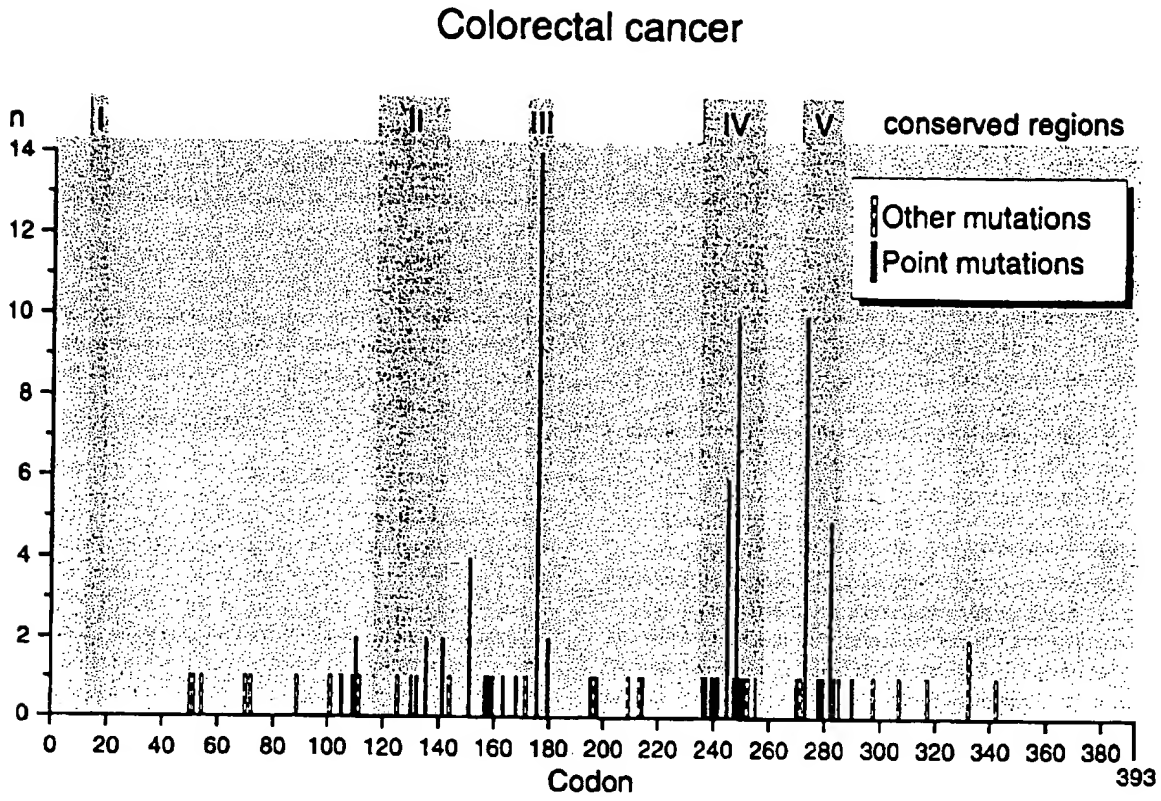


Fig 1. Distribution of missense mutations and other genetic changes along the *p53* gene.

18%). Twenty (22%) of the tumors that displayed the wild-type gene on cDNA sequencing were positive by IHC analysis (Table 2).

p53 *Mutations and Serum* p53 *Antibody Levels*

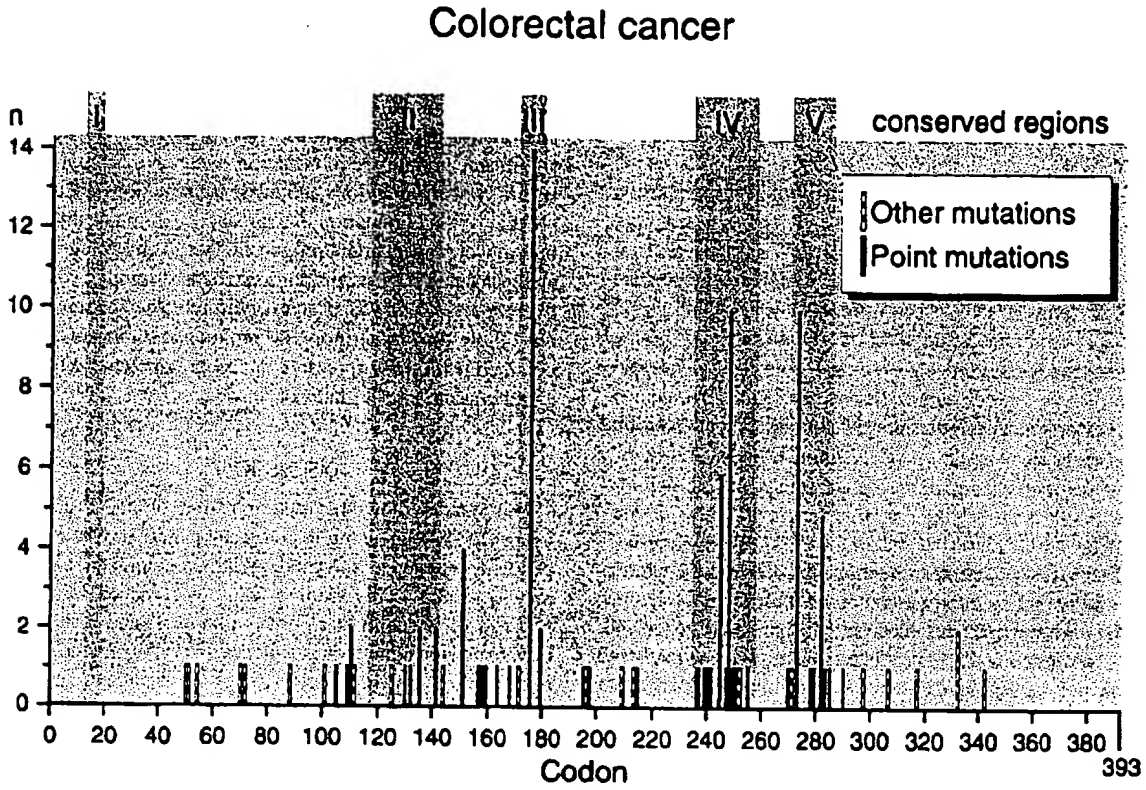
The association of tumors with *p53* genetic changes with increased serum *p53* antibody levels was significant: 23 (50%) of 46 patients with tumors showing genetic changes had high levels of serum *p53* antibodies; this was the case in only two (5%) of 44 patients who did not have *p53* genetic changes ($P = .0002$) (Table 2). The association between *p53* genetic changes and serum *p53* antibody levels was observed in 20 (71%) tumors with missense mutations.

p53 *Mutations and Prognosis*

Patients with *p53* mutations, as determined by cDNA sequencing, had a significantly shorter cancer-specific survival time when compared with patients with the wild-type *p53* gene. This finding was observed when the total patient material was analyzed ($P = .01$, log-rank test; Fig. 2A) but

Table 1. *p53* Mutations in Colorectal Cancer and Their Relation to Age, Sex, Dukes Stage, Tumor Differentiation, and Tumor Localization

	No. of Cases	*p53* Mutation Analysis			p53 IHC Analysis		
		No.	%	P	No.	%	P
Age							
≤ 70 years	91	53	58		43	47	
> 70 years	98	44	45	NS	46	47	NS
Sex							
Male	80	55	50		43	54	
Female	109	42	52	NS	46	42	NS
Dukes stage							
A	29	13	45		15	52	
B	96	51	53		41	43	
C	40	17	42		20	50	
D	24	16	67	NS	13	54	NS
Tumor differentiation							
Good	26	13	50		11	42	
Moderate	128	71	55		65	51	
Poor	35	13	37	NS	13	37	NS
Tumor localization							
Proximal colon	78	28	36		25	32	
Distal colon and rectum	111	69	62	.04	64	58	.04



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Patients with *p53* mutations, as determined by cDNA sequencing, had a significantly shorter cancer-specific survival time when compared with patients with the wild-type *p53* gene. This finding was observed when the total patient material was analyzed ($P = .01$, log-rank test; Fig. 2A) but

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	No. of Cases	*p53* Mutation Analysis		P	p53 IHC Analysis		P
		No.	%		No.	%	
Age							
≤ 70 years	91	53	58		43	47	
> 70 years	98	44	45	NS	46	47	NS
Sex							
Male	80	55	50		43	54	
Female	109	42	52	NS	46	42	NS
Dukes stage							
A	29	13	45		15	52	
B	96	51	53		41	43	
C	40	17	42		20	50	
D	24	16	67	NS	13	54	NS
Tumor differentiation							
Good	26	13	50		11	42	
Moderate	128	71	55		65	51	
Poor	35	13	37	NS	13	37	NS
Tumor localization							
Proximal colon	78	28	36		25	32	
Distal colon and rectum	111	69	62	.04	64	58	.04

Table 2. Comparison of p53 cDNA Sequencing With the p53 IHC Method and With Serum p53 Antibody Levels (S-p53)

	cDNA Sequencing			
	Mutation	Wild-type	Unknown	Total
IHC method				
Positive	70	20	2	92
Negative	28	70	0	98
Unknown	1	0	0	1
Total	99	90	2	191
S-p53 antibody levels				
Positive	23	2	2	27
Negative	23	42	0	65
Unknown	0	0	0	0
Total	46	44	2	92

not when the analysis was restricted to the potentially cured patients in Dukes stages A to C (P = .12, log-rank test; data not shown).

Overexpression of p53 *Protein and Prognosis*

There was no significant relationship between the overexpression of *p53* protein and cancer-specific survival, neither when the total patient material was analyzed (P = .40, log-rank test; Fig. 2B) nor when the analysis was restricted to the potentially cured patients (P = .64, log-rank test; data not shown).

Uni- and Multivariate Analyses

The results of the univariate analyses for *p53* mutations overall and for selected subgroups are shown in Table 3. *p53* mutations overall, *p53* mutations outside conserved regions, and other types of mutations versus wild-type *p53* showed significant prognostic information, whereas *p53* mutations within conserved regions and missense mutations did not.

Age, sex, *p53* mutation, Dukes stage, and tumor differentiation were independent prognostic factors in a multivariate analysis using the Cox proportional hazards model, whereas overexpression of *p53* protein and tumor localization were not (Table 4). Furthermore, no prognostic information was obtained from overexpression of *p53* protein when tested in a multivariate model (data not shown).

DISCUSSION

Our results show that determination of *p53* mutations using cDNA sequencing in colorectal cancer provides independent prognostic information about colorectal cancer–related deaths. This finding is in accordance with the findings of Hamelin et al,[8] who also evaluated *p53* mutational status in a multivariate model. In two other studies,[9,11] a correlation between *p53* mutations and patient survival, using Kaplan-Meier life table analyses, was also observed.

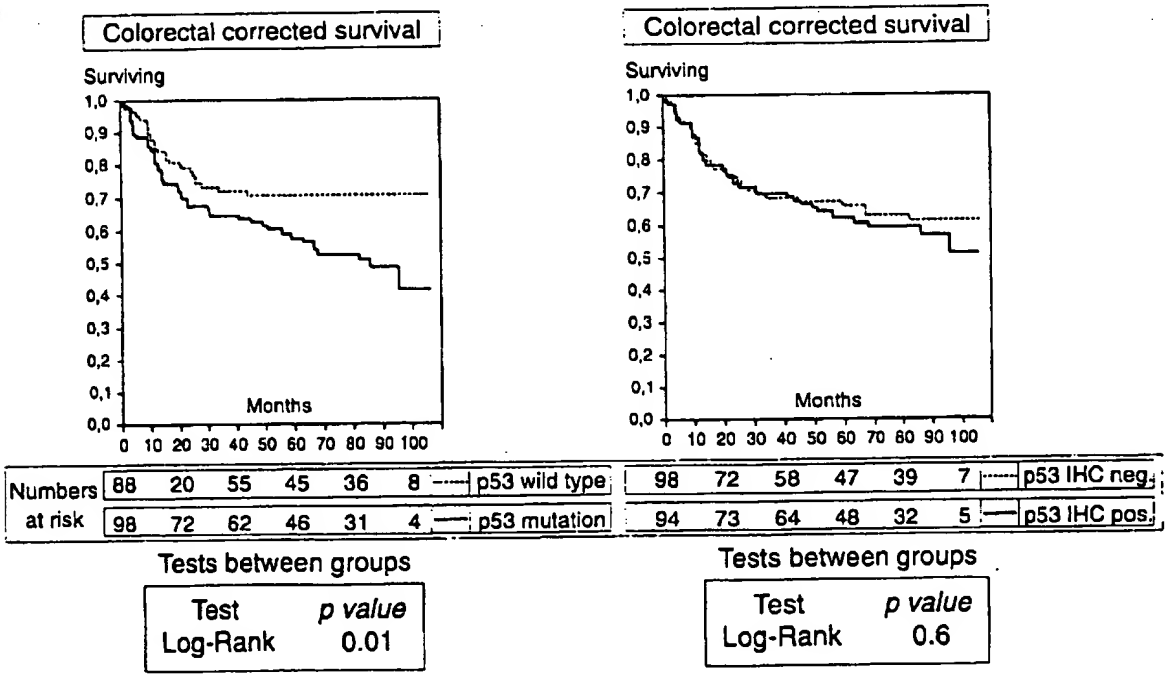

Fig 2. (Left) Life table plots for Dukes stages A to D. The solid line indicates the *p53* mutation (n = 97), and the dotted line indicates wild-type *p53* (n = 90). (Right) Life table plots for Dukes stages A to D. The solid indicates *p53 IHC positivity (n = 90), and the dotted line represents p53 IHC negativity (n = 97). Cancer-specific curves were generated according to the Kaplan-Meier method, and statistical comparisons were made using the log-rank test.*

Table 3. Univariate Analyses of the Effects of *p53* Mutations, Missense Mutations, and Mutations Inside and Outside Conserved Regions

Variable		*P*	RH	Confidence Interval
No. of patients	187			
No. of cancer specific deaths	74			
p53 mutations				
Wild-type			1.0	Ref
Mutation		.04	1.63	1.28-2.10
Missense mutations				
Wild-type			1.0	Ref
Mutation		.15	1.45	0.93-1.88
Types of mutations other than missense mutations				
Wild-type			1.0	Ref
Mutation		.01	2.36	1.40-3.30
Mutations inside conserved regions				
Wild-type			1.0	Ref
Mutation		.20	1.40	0.87-1.83
Mutations outside conserved regions				
Wild-type			1.0	Ref
Mutation		.01	2.23	1.63-3.04

The majority of published studies focusing on *p53* gene status and prognosis in colorectal cancer have been based solely on IHC analyses. Some studies have then shown a correlation with survival[14-17]; this finding could not, however, be confirmed by us[23] or by others.[10,18-20,22] Our results suggest that the choice of methodology could have influenced the outcome of the studies and thus the overall clinical significance of *p53* mutations. Direct comparisons among different IHC studies are difficult. Different mono- and polyclonal antibodies, plus both fresh tissue samples and archival material, have been used. Moreover, there is a lack of consensus as to how to interpret nuclear staining, ie, the number of cells that need to be stained for a tumor to be considered positive for *p53* overexpression. In addition, our present results show that cDNA sequencing gives better prognostic information than IHC assessment of the *p53* protein (relative hazard [RH], 1.63, $P = 0.04$; *v* RH, 1.02, $P = .72$), in agreement with results previously reported for breast cancer.[13]

We are the first to report that mutations that occur outside evolutionary conserved regions seem to be related to a significantly poorer prognosis (RH, 2.23, $P = .01$). This finding is in contrast to that of Goh et al,[9] who stated that missense mutations within conserved regions were associated with the worst prognosis. In that study, however, only exons 4 to 9 in the *p53* gene were studied and not the entire *p53* gene, as was the case in our study. Mutations in the *p53* gene occur predominantly in the segment of the genome that is responsible for DNA binding.[30] This genomic region, coded mostly by exons 5 to 8, is referred to as the evolutionary conserved domain,[31] in which evolutionary conserved regions are located. We found that the frequency of missense mutations was higher inside the conserved regions (82%), which was in agreement with other reports,[9,11] whereas other types of mutations seemed to be more widely distributed.

The concordance between the cDNA sequencing and IHC analysis in our material was only 74%. Although the correlation is highly statistically significant ($P < .001$), it indicates that the two methods have partly different sensitivity for detecting aberrant *p53*. The samples used for IHC analysis and cDNA sequencing were selected from each tumor and located adjacent to each other. Normal intestine was always excluded from the samples, but microdissection was not performed. The possible impact of intratumoral connective tissue was considered negligible, as the tumor tissue always dominates intratumoral stroma demonstrated by IHC analyses. Similar results have been reported in studies comparing *p53* mutations and overexpression of *p53* protein with polyclonal antibody 1801[11,32] or with monoclonal antibody DO-7.[33] As most genetic changes other than missense mutations do not result in the expression of any *p53* protein, it is therefore natural that these genetic changes more frequently fail detection by the IHC method than by cDNA sequencing. This failure indicates a weakness of the IHC method to find these types of mutations, which represent 20% of all mutations in colorectal cancer.[8] In contrast, the IHC method was relatively effective in identify-

Table 4. Multivariate Analysis of the Effects of Age, Sex, *p53* Mutation, p53 Overexpression, Dukes Stage, Tumor Localization, and Tumor Differentiation on Survival in Patients Operated on for Colorectal Cancer

Variable		*P*	RH	Confidence Interval
No. of patients	187			
No. of cancer specific deaths	74			
Age		.01	1.03	1.02-1.17
Sex				
Male			1.0	Ref
Female		.02	0.6	0.44-0.72
p53 Mutational analyses				
Wild-type			1.0	Ref
Mutation		.03	1.7	1.35-2.22
p53 of IHC analyses				
Wild-type			1.0	Ref
Overexpression		.71	1.02	0.84-1.65
Dukes stage				
A			1.0	Ref
B		.04	3.6	1.96-6.67
C		.001	8.9	4.79-16.6
D		.0001	58.1	30.6-110.6
Tumor localization				
Colon			1.0	Ref
Rectum		.95	0.96	0.87-1.38
Tumor differentiation				
Good			1.0	Ref
Moderate		.04	3.0	1.78-5.08
Poor		.02	4.0	2.24-6.93

ing missense mutations (81%), which may be due to the fact that these mutations result in excessive *p53* protein expression not sufficiently counterbalanced by Mdm2.[34,35] The cDNA sequencing method used in this study has previously been used for the detection of *p53* changes in breast cancer.[13] We have had no indication that the method generated false-positive results. It could theoretically be the case as a consequence of contamination of the samples. Stringent procedures during the pre-PCR phase of the analysis, in combination with appropriate negative controls, reduced to a minimum the risk of obtaining false-positive results.

Baas et al[36] showed that strong rather than weak IHC staining is associated to a great extent with *p53* genetic changes detected by gene analyses. Twenty tumors with no detectable *p53* changes were positive according to IHC analysis, thus possibly indicating an overexpression of the *p53* protein without genetic changes. In theory, this may be caused by an inability to detect certain mutations. However, cDNA sequencing is a very sensitive method, and the frequency of missed mutations is extremely low.[13] The theory of stabilization of the *p53* protein is more likely, possibly involving a nonmutational pathway responsible for the overexpression of wild-type *p53* protein.[37]

False-negative results can occur putatively, if tumor samples used for the analyses contain a low proportion of tumor cells in relation to normal cells. As a result, false-negative results dilute the signal corresponding to mutation below the threshold of detection of cDNA sequencing. The biopsy specimens selected for this study were carefully chosen to contain abundant tumor cells, which can explain the strong signal seen in virtually all biopsy specimens.

In this study, we were also able to demonstrate a significant association between *p53* genetic changes in tumor sections, as determined by cDNA sequencing, and elevated levels of serum *p53* antibodies ($P = .0002$), although increased levels were seen in only 50% of the patients with *p53* mutations. Missense mutations were more commonly associated with high serum *p53* antibody levels (71%). A missense mutation results in an accumulation of mutant *p53* protein, and this seems to be necessary for the development of a humoral response with detectable levels of anti-*p53* antibodies in the peripheral circulation.

Fluorouracil is a cell cycle–specific cytotoxic agent used in colorectal cancer that induces DNA damage, both by inhibition of thymidylate synthase and by direct fragmentation of DNA. The tumor cell may respond to DNA damage by undergoing G_1 arrest or by inducing cell death by apoptosis. This response may rely on a normal *p53* gene and a functional *p53* protein.[38] Thus, it seems that normal *p53* protein is essential for optimal cytotoxic drug action. Lowe et al[39] have shown that the status of the *p53* gene may play an important role in the responsiveness to treatment with cytotoxic drugs. This also seems to be the case for radiotherapy.[40] No patients in our series received adjuvant chemotherapy treatment, which has now become routine in clinical practice for Dukes stage C patients. Whereas normal *p53* protein facilitates apoptosis induced by chemotherapy, *p53* mutation instead leads to chemotherapy resistance[38] and thus to overtreatment in a large number of patients.

Detecting genetic changes in the *p53* gene with cDNA sequencing is technically demanding. It is likely that methods as accurate as cDNA sequencing but requiring fewer technical skills will be used for routine screening of *p53* gene mutations. Such a method has recently been described.[41] Using this method, identification of wild-type samples would only require cDNA sequencing of aberrant samples. This kind of strategy would significantly reduce the amount of work needed to establish the relationship between genotype and phenotype in a clinical context.

In essence, *p53* cDNA sequencing in colorectal cancer provides an accurate and sensitive method for localization of genetic changes. Our observations demonstrate that cDNA sequencing is superior to IHC analysis in this respect. However, if *p53* cDNA sequencing or any other method is to have an impact on clinical practice in the determination of treatment, it must also be possible to analyze preoperative tumor biopsy specimens more efficiently. The question of tumor heterogeneity in relation to biopsy sampling[26] will need further attention before cDNA sequencing can be used routinely.

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Table 1. The relation between TNM, Astler-Coller, and Dukes' stage

TNM STAGING	ATLER-COLLER CLASSIFICATION	DUKES' STAGES
T1 Involvement of submocosae but not muscularis propria T2 Invasion into but no penetration through muscularis propria	A Involvement of submucosae, but not muscularis propria B1 Invasion into, but no penetration through muscularis propria	A Tumour has not penetrated beyond muscularis propria
T3 Penetration through muscularis propria and into serosa or pericolic fat, but not into free peritoneal cavity or other organs T4 Invasion of other organs or involvement of free peritoneal cavity	B2 Penetration through muscularis propria and into serosa or pericolic fat; no nodal involvement B3 Invasion of other organs; no nodal involvement	B Tumour has penetrated beyond muscularis propria; no nodal involvement
N0 No nodal involvement N1 1-3 pericolic/perirectal nodes involved N2 ≥4 pericolic/perirectal nodes involved N3 Any regional nodes along a named vascular trunk involved	C1 No penetration through muscularis propria; nodal involvement C2 Penetration through bowel wall; nodal involvement C3 Invasion of other organs; nodal involvement	C Lymph node involvement
M0 No distant metastases M1 Distant metastases	D Distant metastases	D Distant metastases

Table 2. Dukes' stage and survival.

Stage	Proportion(%)	5-year survival(%)
A	15	80-100
B	40	40-65
C	35	25-45
D	10	0-5

Arhand Lig Kressner

p53 Immunoreactivity and Single-Strand Conformational Polymorphism Analysis Often Fail To Predict *p53* Mutational Status

Dawn M. Tolbert, Ph.D., Amy E. Noffsinger, M.D., Mary A. Miller, B.S., Gary W. DeVoe, B.S., Grant N. Stemmermann, M.D., John S. Macdonald, M.D., Cecilia M. Fenoglio-Preiser, M.D.

Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio (DMT, AEN, MAM, GWD, GNS, CMF-P), and the Department of Hematology and Oncology, The Saint Vincent's Comprehensive Cancer Center, New York City, New York (JSM)

The intent of this study was to investigate the ability of p53 expression and single-strand conformational polymorphism analysis (SSCP) to predict *p53* mutational status in archival, paraffin-embedded tissues of gastric cancer. We evaluated paraffin-embedded tissues from 78 patients with advanced gastric cancer. The mutational status of the *p53* gene (exons 5–9) was examined by SSCP analysis and by direct sequencing. These results were compared with p53 expression as assessed by immunohistochemical analysis (IHC). We graded p53 expression on a scale from 0 to 8 on the basis of both the intensity and the number of cells staining. Overall, we detected p53 immunoreactivity in 75.6% of the gastric cases; 19 (32.2%) of these cases scored from 1 to 4, and 40 (67.8%) cases scored from 5 to 8. *p53* gene mutations were detected in 18 cases (23.1%) by SSCP and in 28 cases (36%) by direct sequencing. Thus, SSCP failed to detect 38% of the mutations found by sequencing. The majority of missed mutations involved exons 7 and 8. The concordance between IHC and SSCP was 37%, and the concordance between IHC and direct sequencing was 50%. Forty-five percent of cases positive by IHC failed to show mutations in exons 5 through 9. Five percent of cases negative by IHC (4 cases) contained mutations. One had a 1-base pair insertion; one had a mutation that resulted in a stop codon; the third had a mutation in exon 8; and the fourth had a mutation in both exons 5 and 8. Our findings indicate that p53 immunoreactivity correlates with th presence or absence of gene mutations in 50% of advanced gastric cancers when exons 5 through 9 are examined and that IHC cannot be reproducibly used as a marker of mutation in the most commonly mutated exons of the *p53* gene. Furthermore, the sensitivity of SSCP for detecting mutations is only 62%. Thus, SSCP analysis cannot be used reliably to screen for *p53* mutations.

KEY WORDS: Immunohistochemistry, Mutation, p53, SSCP.

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The *p53* gene, located on the short arm of chromosome 17, encodes a protein that plays a critical role in cell cycle regulation and tumor suppression. This "guardian of the genome" causes G1 cell cycle arrest in response to DNA damage (1, 2). This allows the injured cell time to repair the damaged DNA before reinitiating replicative DNA synthesis and/or mitosis. When normal p53 functions are lost, either through mutation, genetic loss, or binding of proteins to the wild-type p53 protein, the cell fails to undergo cell cycle arrest in response to DNA damage, and the cell either dies or continues to proliferate, passing the genetic damage on to its progeny (3). The *p53* gene is also likely to play a critical role in the response of many cancers to chemotherapy (4). Many chemotherapeutic agents induce programmed cell death, presumably via *p53*-dependent pathways (4). Our long-term goal is to assess the impact of *p53* mutations in patients response to chemotherapy. To do this, it is important to identify the most reliable method of detecting *p53* mutations. Three approaches are commonly used to analyze *p53* mutations: immunohistochemical analysis (IHC), single-strand conformational polymorphism analysis (SSCP), and direct sequencing of the exons, usually exons 5 through 9. A careful anal-

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Address for reprints: Operations Office, Southwest Oncology Group (SWOG-9008), 14980 Omicron Drive, San Antonio, TX 78245-3217.
Address reprint requests to: Cecilia M. Fenoglio-Preiser, M.D., Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0529; e-mail: fenoglio@ucbeh.san.uc.edu; fax: 513-558-2289.

ysis however, of the concordance of mutation detection among these methods has not been performed. Thus, we found it necessary to identify the simplest and most accurate way to detect the presence of mutations in paraffin-embedded tissues derived from patients originating in multiple institutions.

MATERIALS AND METHODS

Tissue Samples

We examined formalin-fixed, paraffin-embedded tissues from 78 patients who underwent gastrectomy for advanced gastric cancer. All of these patients were enrolled in the prospective intergroup study coordinated by the Southwestern Oncology Group (SWOG 9008) (INT 0116). Both tumor and nonneoplastic tissues were obtained from each of these patients. The *p53* gene status was analyzed by IHC, SSCP, and direct sequencing in all of the 78 cases.

IHC

p53 protein expression was evaluated by IHC with use of the monoclonal antibody (clone DO-1; Oncogene Science, Cambridge, MA) at a working concentration of 1:300 dilution. Initially, we compared clone DO-1 with clone PAB 1801, BP 53-12, and DO-7 before deciding to use the DO-1 clone. All of the above antibodies yielded similar results. Clone DO-1 antibody reacts with both wild-type and mutant forms of the p53 protein. Freshly cut sections 4 μm thick mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA) were dried overnight at 60° C, deparaffinized in xylene, and rehydrated through decreasing concentrations of ethanol to water. Epitope unmasking was accomplished by simmering at 60% power in 10 nM citrate buffer at pH 6.0 for 15 minutes after coming to a full boil (7.5 min) in a 900-W Sharp (Mahwah, NJ) Carousel II microwave oven. Slides were cooled for 30 minutes at room temperature in the citrate buffer before proceeding. The slides were then microwaved in 0.01 M citrate buffer, pH 6.0, using a standard technique. Immunohistochemical staining was performed using an indirect biotin avidin method on a Ventana 320ES automated immunostainer (Ventana Medical Systems, Tucson, AZ). The stained sections were lightly counterstained with nuclear fast red and examined. Identically pretreated negative controls on serial sections of the gastric tumors were run concurrently, using purified nonimmune mouse immunoglobulin G at 2 μg/mL in place of the primary antibody. The positive controls consisted of a colon tumor sausage prepared by us that contains 20 different colon tumors. This control was included in each run. Immunohistochemically negative cases were repeated to test whether antigenicity was destroyed.

A combined qualitative and quantitative approach was used to assess both the intensity and percentage of p53-immunoreactive cells. Quantitatively, the proportion of p53-positive tumor cells was scored as follows: 4+ when 76 to 100% of tumor cells were positive; 3+ when 51 to 75% of tumor cells were positive; 2+ when 26 to 50% of tumor cells were positive; 1+ when fewer than 25% of tumor cells were positive; and 0 when no tumor cells were positive. Qualitatively, staining intensity was scored as follows: 4+ for the most intensely positive staining; 3+ for moderately intense positive staining; 2+ for moderately positive staining; 1+ for faintly positive staining; and 0 for no p53 staining. The overall p53 protein expression score was based on the sum of the quantitative and the qualitative scores. Possible IHC scores were 0 and 2 through 8 (because the final score represents a total of the qualitative and quantitative scores, a score of "1" was impossible). The slides stained for IHC were independently read by three of the authors (GNS, AEN, CF-P). The agreement between the three in the final score was 95%. In case of disagreement, the three evaluators examined the slides together and decided a consensus score. IHC was performed and interpreted before the sequencing or SSCP analysis was complete, and the data were entered into a database (Access; Microsoft, Bellevue, WA) by one of the authors (GWD).

Tissue Microdissection

Seven to ten serial sections 5 μm thick were cut and applied to glass slides. The first slide was stained with hematoxylin and eosin for histologic evaluation. With a marking pen, the protocol pathologist (GNS) circled the areas containing cancerous and noncancerous tissue, and cancerous areas were then microdissected from noncancerous area of the unstained slides.

DNA Extraction

Tumor DNA and corresponding non-neoplastic DNA were extracted from the microdissected paraffin sections by a standard proteinase K digestion procedure. Briefly, the samples were deparaffinized in xylene, washed with 100% ethanol, and dried in a speed vacuum. The dried samples were resuspended in 100 μL digestion buffer (50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20) containing 200 μg/μL proteinase K and incubated at 37° C overnight. The samples were then heated at 95° C for 10 minutes to inactivate the proteinase K and stored at −20° C for later use in the polymerase chain reaction (PCR) studies. A 1-μL aliquot of the superna-

tant containing genomic DNA was used for the p53 studies.

SSCP

Briefly, amplification was performed in 1× PCR buffer [10 mM Tris–hydrochloric acid (pH 8.3), 50 mM potassium chloride], 2 mM magnesium chloride, 6 mM of each primer, 100 μM dATP, 200 μM each of dCTP (deoxycytosine triphosphate), dGTP (deoxyguamine triphosphate), and dTTP (deoxythymine triphosphate), 2.5 μCi [α-^{32}P]dATP (NEN, 10 μCi/μL, 3000 Ci/mmol), 4% dimethylsulfoxide, and 0.6 units of Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, CT) in a total volume of 25 μL. The sequences of the oligonucleotide primers used to amplify exons 5 through 9 of the *p53* gene are listed in Table 1. Exons 5 through 9 were chosen for analysis because this region of the *p53* gene contains the mutational "hot spots." After an initial hot start, amplification was performed for 35 cycles of denaturation at 94° C, with annealing at 58° C (exons 5, 6, 9), 55° C (exon 8), or 60° C (exon 7), and extension at 72° C in a DNA Thermal Cycler (MJ Research, Watertown, MA). Two microliters of each amplified product were mixed with 8 μL of loading buffer (95% formamide, 10 mM sodium hydroxide, 0.25% bromophenol blue, 0.25% xylene cyanol). The samples were then denatured by heating at 94° C and immediately chilled on ice. A 5-μL aliquot of each sample was loaded onto a 0.5× MDE gel (AT BioChem, Malvern, PA) and run at 10 W for 8.5 to 14 hours at either room temperature or 4° C (depending on the exon). The gel was exposed to Kodak (Rochester, NY) Biomax MR autoradiography film without an intensifying screen for 1 to 2 days at −80° C. A negative control containing no template DNA and a positive control (a cell line harboring a known point mutation) were run in parallel for each amplification reaction. For positive controls, we used the cell lines SK-BR-3, T-47D, DAOY P5, MDA-MB-468, and SW-480 because they contain known point mutations in exons 5 through 9, respectively. All of the mutations were verified by direct sequencing. The SSCP was read by one of the authors (DMT) who had no knowledge of the IHC results. The data were then given to GWD to enter into the database.

TABLE 1. Oligonucleotide Primers for p53 Analysis

Exon	Upstream	Downstream
5	TTCCTCTTCCTGCAGTACTC	ACCCTGGGCAACCAGCCCTGT
6	ACAGGGCTGGTTGCCCAGGGT	AGTTGCAAACCAGACCTCAG
7	GTGTTGTCTCCTAGGTTGGC	GTCAGAGGCAAGCAGAGGCT
8	TATCCTGAGTAGTGGTAATC	AAGTGAATCTGAGGCATAAC
9	GCAGTTATGCCTCAGATTCAC	AAGACTTAGTACCTGAAGGGT

Direct Sequencing of PCR Products

The PCR was performed with 1 μL of genomic DNA, 10 mM Tris–hydrochloric acid (pH 8.3), 50 mM potassium chloride, 2 mM magnesium chloride, 6 mM of each primer, 200 μM dNTPs (deoxynucleoside 5′-triphosphates), 4% dimethylsulfoxide, and 0.6 units of Amplitaq DNA polymerase (Perkin-Elmer). The amplification conditions and primers used were as described above. Direct sequencing of the amplified products was performed with a thermosequenase radiolabeled terminator-cycle sequencing kit (Amersham Life Sciences, Cleveland, OH). The sequencing reactions were performed according to the manufacturer's instructions. In cases in which an unusual sequencing pattern was observed, DNA from the abnormally migrating bands (detected by SSCP) was eluted from the MDE gel and subjected to asymmetric PCR (5); sequencing reactions were then performed. All of the sequencing gels were read by DMT, who had no knowledge of the *p53* IHC results. Random gels were also read by AEN and CF-P. The data were then transmitted to GWD for entry into the patient database.

RESULTS

Incidence of p53 Protein Expression

The p53 antibody stained a subset of the gastric caners. Rare isolated positive cells could be identified in the normal gastric epithelium but in no other cell types. Positive staining was also present in a percentage of stomachs containing intestinal metaplasia. The positive and negative controls were appropriately positive and negative. The p53 immunohistochemical staining pattern varied considerably among the gastric cancer cases (Table 2). Overall, using the scoring system described, 59 (75.6%) of 78 cases were positive by IHC and 19 (24.4%) of 78 cases were negative. Nineteen (24.4%) of 78 cases had an immunohistochemical score of 1 to 4, and 40 (51.3%) of 78 cases had an immunohistochemical score of 5 to 8.

TABLE 2. Frequency of p53 Protein Expression

IHC score	No. of cases (%)
0	19 (24)
2	10 (13)
3	6 (8)
4	3 (4)
5	7 (9)
6	10 (13)
7	6 (8)
8	17 (22)
Total	78 (100)

IHC, immunohistochemical.

Incidence of *p53* Gene Mutation by SSCP

p53 mutations were detected in 18 cases (23.1%). Mutations were found in exons 5, 7, and 8 by this detection method. No mutations were found in exons 6 and 9 (Table 3).

Incidence of *p53* Gene Mutation by Sequencing

There were 29 *p53* gene mutations found among the 78 cases (36%). Most mutations affected exons 5, 7, and 8; none were detected in exons 6 and 9. One case (Case 7) had mutations in exons 5 and 8. Insertions and/or deletions were only observed in exon 7 (Cases 33, 39, and 71). A missense mutation resulting in a change of the amino acid sequence was present in 24 (96%) of the 25 remaining cases. G:C to A:T mutations were detected in 14 (56%) of these 25 cases. Other mutations included C:G to T:A transitions in eight cases, G:C to T:A transversions (Cases 63 and 76), and a T:A to A:T transversion (Case 31) (Table 3). Only one case (Case 50) had a nonsense mutation resulting in the production of a stop codon. As shown in Figure 1, the distribution of mutations found in the p53 protein revealed the presence of several mutational hotspots at codons 175, 273, and 282.

Concordance Between p53 *Mutation by SSCP Analysis and Direct Sequencing*

Direct DNA sequencing was performed in all of the cases. *p53* mutations were detected by direct sequencing in 36% of the cases, contrasting with mutations found in 23.1% of cases by SSCP. This means that the SSCP reactions failed to show a mobility shift in the gel and were falsely negative in 13% of all the cases and 38% of the mutated cases. The majority of the missed mutations involved exons 7 and 8.

Concordance Between p53 Immunoreactivity and Gene Mutation

To evaluate whether immunohistochemical detection of p53 correlated with the presence of *p53* gene mutations in exons 5 through 9, we compared the results from direct sequencing and immunochemical staining (Tables 4 and 5). Those tumors lacking p53 overexpression and mutation (IHC−/mutation−) or those having both p53 expression and mutation (IHC+/mutation+) (Fig. 2) were classified as showing concordance. Fifteen (19%) of the 78 tumors had no *p53* mutations detectable by either IHC or sequencing. Twenty-four tumors (31%) were positive for mutation by both p53 immunoreactivity and genetic analysis. Of these 24 tumors, mutations were detected in 1 (4.2%) assigned to the 1-through-4 immunohistochemical score group and 23 (96%) assigned to the 5-through-8 score group, suggesting that mutations are more common in tumors with a higher immunohistochemical score.

Those tumors showing p53 expression in the absence of mutation (IHC+/mutation−) or mutation

TABLE 3. p53 Mutations in Advanced Gastric Cancer

Patient no.	Case	IHC score	SSCP/exon	Codon	Nucleotide mutation	Amino acid substitution
	147086	7	8 (seq+/sscp−)	273	CGT → CAT	Arg → His
4	144729	6	8 (seq+/sscp−)	273	CGT → TGT	Arg → Cys
5	146103	0	8	282	CGG → TGG	Arg → Trp
6	147179	6	8	273	CGT →TGT	Arg → Cys
7	140812	0	5	156	CGC → TGC	Arg → Cys
7	140812	0	8 (seq+/sscp−)	282	CGG → TGG	Arg → Trp
10	147034	8	5	175	CGC → CAC	Arg → His
15	139917	8	7	245	GGC → AGC	Gly → Ser
17	142535	8	8	273	CGT → CAT	Arg → His
20	140805	8	8 (seq+/sscp−)	273	CGT → CAT	Arg → His
24	140172	8	5	135	TGC → TAC	Cys → Tyr
26	139852	5	7	258	GAA → AAA	Glu → Lys
31	137029	5	5	147	GTT → GAT	Val → Asp
32	133590	8	8 (seq+/sscp−)	282	CGG → TGG	Arg → His
33	140416	5	7		Deletion 3 bp	
34	140495	8	5	175	CGC → CAC	Arg → His
39	39521	8	7		Deletion 3 bp	
43	136384	7	7 (seq+/sscp−)	248	CGG → CAG	Arg → Gln
45	140883	8	5 (seq+/sscp−)	176	TGC → TAC	Cys →Tyr
46	140915	8	7 (seq+/sscp−)	241	TCC → TGC	Ser → Cys
50	139770	0	5	146	TGG → TAG	Trp → *stop*
51	147794	8	7 (seq+/sscp−)	241	TCC → TTC	Ser → Phe
56	147773	2	8 (seq+/sscp−)	282	CGG → TGG	Arg → Trp
61	142304	7	7 (seq+/sscp−)	237	ATG → ATA	Met → Ile
63	139242	6	5	176	TGC → TTC	Cys → Phe
71	148029	0	7		Insertion 1 bp	
74	149130	8	8	285	GAG → AGG	Glu → Lys
75	150137	8	5	175	CGC → CAC	Arg → His
76	139957	6	7	245	GGC → TGC	Gly → Cys

IHC, immunohistochemical; SSCP, single-strand conformational polymorphism.

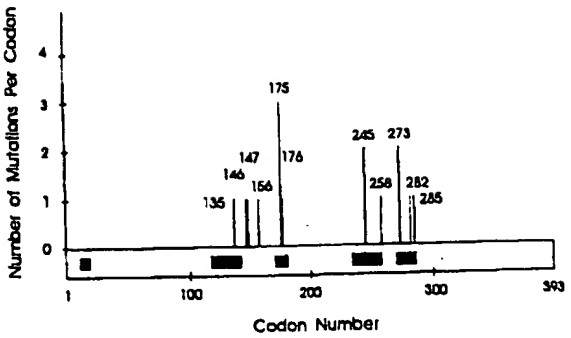

FIGURE 1. Diagram showing the mutational hot spots for the gastric cancers analyzed in this study.

TABLE 4. Summary of p53 Expression and Gene Mutation

IHC score	No. of mutations (%)	Mutations at exons 5–9 (%)	Total (%)
0	15 (19)	4 (5)	19 (24)
2	9 (12)	1 (1)	10 (13)
3	6 (8)	0 (0)	6 (8)
4	3 (4)	0 (0)	3 (4)
5	4 (5)	3 (4)	7 (9)
6	6 (7)	4 (5)	10 (13)
7	3 (4)	3 (4)	6 (8)
8	4 (5)	13 (17)	17 (22)
Total	50 (64)	28 (36)	78 (100)

IHC, immunohistochemical.

in the absence of p53 overexpression (IHC−/mutation+) were classified as being discordant (Fig. 3). Thirty-five (45%) of the 78 tumors showed a positive p53 immunostain but did not contain any mutations. Eighteen of these tumors had an immunohistochemical score between 1 and 4, whereas 17 tumors had an immunohistochemical score of 5 through 8. Examination of the *p53* gene also revealed mutations in exons 5, 7, and 8 that were not detected by IHC (Cases 5, 7, 50, and 71). The discordance observed in Case 50 is typical for a tumor harboring a nonsense mutation that results in a stop codon and premature termination of protein synthesis. Overall, the level of concordance between p53 immunoreactivity and the presence or absence of mutation in advanced gastric cancer was only 50% (Table 5). Conversely, 50% of the tumors showed no concordance.

DISCUSSION

p53 is a tumor suppressor gene, so its inactivation plays an important role in carcinogenesis. p53 abnormalities, including protein overexpression, presence of missense mutations, frameshifts, and loss of heterozygosity, have been widely observed in gastric carcinoma by both immunohistochemical and molecular biologic techniques (6–14). Results between studies, however, vary considerably, depending on the detection techniques used. To our knowledge, this is the only study of p53 alterations in advanced gastric cancer in which p53 expression and mutational status, as assayed by SSCP analysis and direct sequencing, were analyzed simultaneously. Our aim was to identify the frequency of alterations found by each technique and to evaluate the level of concordance (IHC+/mutation+ and IHC−/mutation−) between p53 expression and the presence or absence of gene mutation in these tumors. This information will then be used to decide which technique most easily and most reliably detects *p53* mutations in a large number of similar samples.

A high prevalence (75.6%) of p53 immunoreactivity was observed in the 78 gastric cancer cases studied. Our rate of immunoreactivity is higher than others previously published (6–8). In two separate Japanese studies, immunoreactivity for the p53 protein was detected in approximately 40% of advanced gastric cases (6, 8). Martin *et al.* (7) reported that 57% of gastric carcinomas from European patients expressed high levels of the p53 protein. Our use of an antigen retrieval method could account for the increased frequency of p53 immunoreactivity observed in our patient population, because antigen retrieval methods substantially alter the threshold at which the protein is detected and can increase the number of p53-immunoreactive cells (15, 16). It is also possible that wild-type p53 protein accumulates at levels sufficient to be detected by IHC as a result of either stabilization of the gene product by an interaction with viral or cellular proteins, alterations of the normal degradation process, or a response to DNA damage (17, 18). These factors might differ in different patient populations and in different conditions.

Variations in the immunohistochemical detection of p53 among different studies might also be influenced by other variables, including the sensitivity of the immunoassay, the choice of primary antibody used, differences in interpretation of the staining results, differences in the staining methods, and differences in the length and type of fixation. Baas *et al.* (17) noted substantial variability in IHC sensitivity among six different antibodies used to detect p53 protein. One of the antibodies reported by Baas *et al.* (17) to have a low sensitivity (PAB 1801) was used in both Japanese studies (6, 18). Thus, the lower percentage of p53 immunopositivity observed in these two studies could result from the choice of the antibody. The DO-1 monoclonal antibody used in the current study was not one of the antibodies examined by Baas *et al.* (17) (we compared p53 staining in a series of samples

TABLE 5. Concordance Between p53 Expression and Gene Mutation by Sequencing or Single-Strand Conformational Polymorphism

Method	No. concordant			No. discordant		
	IHC+/M− (%)	IHC−/M− (%)	Total (%)	IHC−/M− (%)	IHC−/M+ (%)	Total (%)
SSCP	14/78 (18)	15/78 (19)	29/78 (37)	45/78 (58)	4/78 (5)	49/78 (63)
SEQ	24/78 (31)	15/78 (19)	39/78 (50)	35/78 (45)	4/78 (5)	39/78 (50)

IHC, immunohistochemical analysis; M, mutation; SSCP, single-strand conformational polymorphism; SEQ, sequencing.

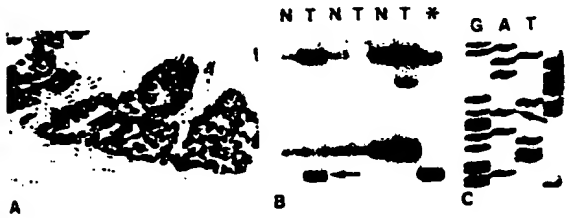

FIGURE 2. IHC (A), SSCP (B), and direct sequencing (C) of *p53*. The IHC shows strong reactivity with the antibody. The SSCP shows a mobility shift (*arrow*) when non-neoplastic tissue (*N*) is compared with tumor tissue (*T*). The * refers to a control sample containing a known mutation in this exon. Direct sequencing for the four base pairs (GATC) shows a mutation from G to A as indicated by the *arrow*. These results are all concordant with one another.

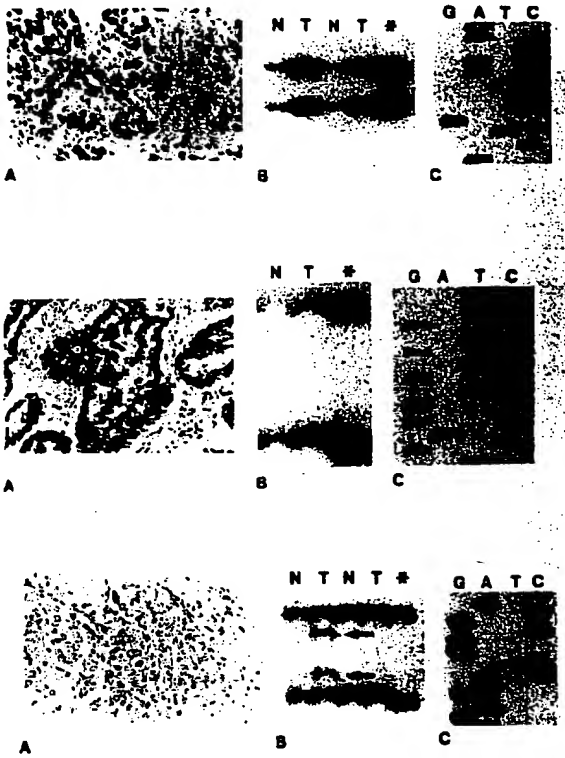

FIGURE 3. IHC (A), SSCP (B), and direct sequencing (C) of *p53* in three tumors showing discordance between the results. The ***top panel*** illustrates a tumor that expresses p53 protein but fails to show a *p53* mutation either by SSCP or by direct sequencing. The ***alphabetical designations*** on (B) and (C) in each of the panels are identical to those shown in Figure 2. The ***middle panel*** shows a tumor (Case 45) that strongly expresses the p53 protein, fails to shift on SSCP, and shows a C-to-T mutation by direct sequencing. The ***lower panel*** illustrates a tumor (Case 7) that fails to express the p53 protein but shows a mobility shift on the SSCP gel, as indicated by the ***arrows*** and the presence of a mutation by direct sequencing with the substitution of a T for a C.

with DO-1, PAB 1801, DO-7, and BP 53-12). Unlike Baas *et al.* (17), we found no differences in the staining of these tumors using our methods (unpublished observations). Therefore, we do not think that the choice of the antibody was a factor in this study. Interpretation of p53 immunostaining results can also be a source of variation. For instance, some investigators considered immunoreactivity to be present if *any* nucleus was positive (6–8). We used a combined qualitative-quantitative approach by taking into account both the p53 signal intensity and the number of p53-positive cells.

In this study, we detected *p53* gene mutations by SSCP in only 23% of all of the advanced gastric carcinomas and in 62% of the mutated tumors. Published reports concerning the incidence of *p53* gene mutations in advanced gastric cancer are limited in number. In one study, Uchino *et al.* (18) reported that *p53* mutations detected by SSCP were present in 25 (42%) of 59 advanced gastric cancers, an incidence higher than that observed in the present study. Epidemiologic differences, the use of different types of tissues (frozen *vs.* paraffin embedded), contamination of tumor samples with nontumorous cells, sensitivity of the SSCP, and the histologic type of cancer present in different patient populations might account for the variations observed between these two studies.

Examination of the status of the *p53* gene by IHC, SSCP, and DNA sequencing reveals discrepancies between p53 immunopositivity and the presence of *p53* gene mutations (Tables 4 and 5). In our study of 78 gastric tumors, mutations in the *p53* gene were detected in 4 (5%) tumors that were negative for p53 by IHC. On the other hand, 35 tumors (45%) showing positive p53 immunoreactivity contained no mutations in exons 5 through 9. These results show a high level of discordance (50%) between the presence of p53 immunoreactivity and the presence of a genetic mutation and cautions against the assumption that a positive IHC indicates the presence of *p53* point mutations. Our findings clearly indicate that p53 immunodetection does *not* correlate with gene mutation and indeed might overestimate the number of tumors containing *p53* mutations.

The high proportion of false-positive (IHC+/mutation−) cases might be caused by an undetected mutation. Most studies reporting on mutations in the *p53* gene only examine exons 5 through 8. There are few reported studies in other tumor systems in which the entire coding region f the *p53* gene was examined. In one such study on breast cancer, Hartmann *et al.* (19) found a number of mutations located outside of exons 5 through 8. These mutations would have been missed if the screening methods were restricted to these exons only. In the present study, the SSCP and DNA sequencing were restricted to exons 5 through 9. Although this region harbors most mutations, it remains possible that advanced gastric cancer might contain *p53* mutations in exons 1 through 4 and 10 through 11. We will explore this possibility in future studies.

Although a number of studies attest to the high sensitivity (> 90% in a fragment of 200 bp) of SSCP (20, 21), we found 11 mutations by direct sequencing that were not detected by SSCP. The sensitivity of SSCP for detecting mutations is only 62%. Likewise, IHC might yield false-negative (IHC−/mutation+) results by missing deletions that will abolish p53 protein production. In addition, some point mutations might result in proteins in which the half-life is not lengthened so that it is not stable enough to be detectable by IHC.

In summary, we demonstrated in this study that the use of IHC alone for the detection of *p53* abnormalities both over-represents and under-represents the presence of *p53* mutations in the mutational hot spot region. At present, we do not have a clear explanation for the immunoreactivity in tumors lacking mutations; additional studies are underway to understand the discrepancy. It is also important to note that the common practice of using SSCP to screen for the presence of mutations and then to use direct sequencing to confirm and characterize these mutations only has a 62% sensitivity for detecting mutations, so 38% of tumors containing mutated p53 might be missed.

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